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Direct Chemical Evidence for a Supramolecular Protein Structure in the Nuclear Envelope

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DIRECT CHEMICAL EVIDENCE FOR A SUPRAMOLECULAR
PROTEIN STRUCTURE IN THE NUCLEAR ENVELOPE

by

David Lee Cochran

B.A., University of Virginia, 1975

Thesis

submitted in partial fulfillment of the requirements for the

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Medical College of Virginia

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Richmond, Virginia

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ABSTRACT

DIRECT CHEMICAL EVIDENCE FOR A SUPRAMOLECULAR PROTEIN STRUCTURE IN THE NUCLEAR ENVELOPE

David Lee Cochran

Medical College of Virginia - Virginia Commonwealth University,
1977

Major Professor: Dr. Keith R. Shelton

Protein subunit structure in the nuclear envelope has not been previously described. Covalent crosslinking of polypeptides provides a method for studying the subunit structure of protein-rich systems. In this study, several polypeptide crosslinking methods have been used to determine associations among polypeptides in isolated chicken erythrocyte nuclear envelope. Interchain associations that exist in the isolated membrane were fixed by crosslinking the polypeptides in the isolated nuclear envelope. Crosslinked and uncrosslinked polypeptides were resolved by electrophoresis in a dissociating detergent system. Each agent caused distinctive alterations in the gel pattern. Certain bands diminished and disappeared while new bands of two or more times their molecular weight appeared in a reciprocal

fashion. Most noteworthy was the major peak of approximately 77,000 daltons molecular weight. This peak cross-linked into dimeric species of approximately 160,000 daltons and higher polymeric species. Some components were distinctly unreactive. The preferential crosslinking reactions indicate that the 77,000 dalton species occur in a specific oligomeric arrangement in the native membrane structure.

This thesis by David Lee Cochran is accepted
in its present form as satisfying the thesis requirement for
the degree of

Master of Science

Date:

Approved:

May 4, 1977
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Advisor, Chairman of Graduate
Committee

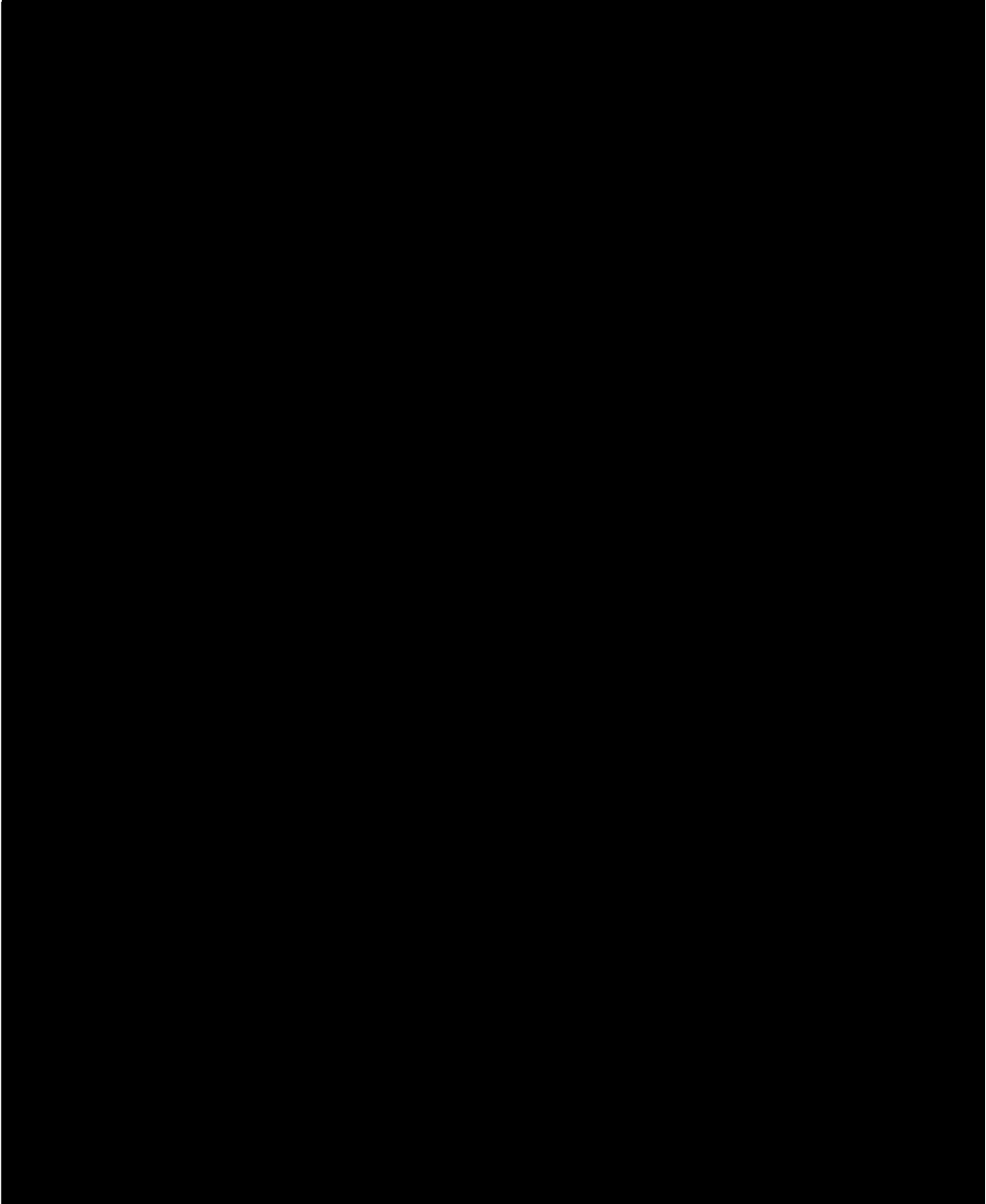
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APPROVED.

Chairman, MCV Graduate Council, Dean, School of
Basic Sciences

Curriculum Vitae





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INTRODUCTION

The feasibility of studying the oligomeric structure of macromolecular assemblies using bifunctional cross-linking reagents was first demonstrated by Davies and Stark (1970). These investigators used a bifunctional crosslinking agent to determine the number and arrangement of subunits in oligomeric proteins. Some of the oligomeric proteins consisted of identical protomers while others were made of different protomers. Crosslinking with bifunctional reagents has since been used in the investigation of a variety of macromolecular structures. These include the study of the topography of components in sub-cellular organelles and membranes, and structure and function relationships in biologically active proteins. Three areas where crosslinking has been extensively applied include ribosomes, histones, and erythrocyte plasma membrane. This technique has been used to examine the spatial relationships that might exist in ribosomes, mainly between the 30S E. coli ribosomal proteins but also between the 50S ribosomal proteins and the initiation factors involved in protein synthesis. Preliminary studies on eukaryotic histones suggested that specific crosslinking reagents would be extremely useful in determining the proximity of

one histone molecule to another. Bifunctional crosslinking reagents have been applied both to isolated histones in solution and to histones within chromatin. A third area is the plasma membrane of the human erythrocyte.

Various reagents have been used in these crosslinking studies. The most widely used crosslinking reagents are the imidoesters. These agents are very specific for primary amino groups in protein (Wofsy and Singer, 1963; Dutton et al., 1966). They react extensively under mild conditions and yield derivatives with unaltered charge. Diimidoesters of various chain lengths can be used in crosslinking proteins. Other crosslinking agents include a class of bifunctional imidoesters in which the two functional imidoester ends of the crosslinking molecule are joined through a disulfide bond. Other useful agents create disulfide bridges between neighboring polypeptides with appropriately spaced sulfhydryl groups.

I. Macromolecular Structures of Discrete Size

A. Enzyme Structure

Aspartate transcarbamoylase, a regulatory enzyme, contains twelve polypeptide chain subunits of two different kinds, six of each. In contrast, some macromolecular structures are polymers of indefinite extent. Acetyl-CoA

carboxylase polymerizes to form long, filamentous protein structures. Moreover, there is often a hierarchy of structural organization. Aspartate transcarbamoylase has been extensively studied. Although this enzyme has twelve polypeptide chains, they are associated such that the enzyme has two catalytic subunits, each having three identical polypeptide chains and three regulatory subunits, each with two identical polypeptide chains different from those in the catalytic subunits. To summarize, macromolecular structures consist of subunits. These subunits may be identical or they may consist of several different kinds of subunits. Also, these subunits may be arranged as repeating units or they may be arranged in some type of hierarchical fashion. Whatever the case may be, it is apparent that a particular macromolecular structure will be composed of subunits and the arrangement of those components will determine the dimensions and properties of the macromolecular structure.

B. Ribosomes

Prokaryotic ribosomes are large particles with a sedimentation coefficient of 70S. They can be dissociated into two subunits with sedimentation coefficients of 30S and 50S. The ribosomal proteins are unusual in comparison with those of other subcellular structures that have been studied in

that the number of different ribosomal proteins is large. Most of them occur in only one copy per ribosome. The 30S subunits from E. coli and B. stearothermophilus each contain 21 distinct proteins, labeled S1 through S21. Whittman and his associates have sequenced many of the ribosomal proteins and have found no duplicated sequences longer than five residues. While sequencing studies may provide pertinent information, it is also essential to know where each component is located within the ribosome, how it interacts with other components, and what its function is.

The ease of disassembly and reconstitution of ribosomes makes possible many experiments to determine where proteins are located within subunits. Specific sites on the ribosome can be modified with chemical reagents. Alternatively individual proteins can be modified and inserted into the subunits as markers. In this way, maps of the ribosome can be assembled. Results obtained from a variety of experiments have been quite consistent. The most common technique for this purpose is the reaction of bifunctional reagents with amino acid residues to link proteins that are adjacent in the intact ribosome. The linked proteins can then be separated from the monomeric proteins and identified.

Sommer and Traut (1975) have identified the components of crosslinked dimers from E. coli 30S ribosomal subunits.

They used a reversible crosslinking reagent, methyl-4-mercaptobutyrimidate. The imidate group of this bifunctional reagent reacts with ribosomal protein resulting in a modified particle containing extra sulfhydryl groups. Mild oxidation then yields intermolecular disulfide crosslinks formed between neighboring proteins. The disulfide crosslinks can be cleaved by mild reduction to regenerate the monomeric components of the crosslinked dimers. The reduction products have the same net charge as the uncrosslinked ribosomal proteins and can be identified by standard electrophoretic techniques. Sommer and Traut, in addition, use a two-dimensional polyacrylamide-dodecyl sulfate diagonal gel electrophoresis technique to separate and identify crosslinked species. The mixture of ribosomal proteins containing species crosslinked by intermolecular disulfide bonds is separated in the oxidized form by electrophoresis in gels containing dodecyl sulfate. The protein in the resulting gel is reduced and electrophoresed in a polyacrylamide-dodecyl sulfate slab gel. The proteins which have not undergone crosslinking fall on a diagonal line. Only proteins previously engaged in protein-protein disulfide bonds fall off the diagonal. The members of specific crosslinked species can be deduced from the molecular weights of the dimers and monomers, and

unambiguous identification of the protein pairs can be made by standard techniques (Sommer and Traut, 1975; Sommer and Traut, 1974; Sun et al., 1974). Application of this and similar diagonal gel techniques to the 30S ribosomal subunit has resulted in the identification of numerous neighboring protein dimers. These dimers include: S2-S3, S3-S4, S3-S10, S4-S6, S4-S8, S4-S9, S4-S12, S4-S13, S5-S8, S5-S9, S6-S18, S7-S9, S13-S19, and S18-S21.

Initiation of protein synthesis in E. coli is mediated by three protein factors IF1, IF2, and IF3 and involves their binding to specific regions of the 30S ribosomal subunit. Bifunctional reagents may be used to identify ribosomal proteins which comprise the binding sites for protein ligands that interact with the ribosome. These reagents have the advantage of avoiding extensive modification of the protein ligand prior to binding (Heimark et al., 1976). This general approach has been employed by Bollen et al. (1975) to demonstrate IF2 initiation factor covalently attaching to the ribosomal proteins S1, S2, S11, S12, S13, S14, and S19.

The 30S subunit appears to contain a single specific site for IF3 which is composed of a segment of 16S RNA and certain 30S proteins. Using N,N¹-o-phenylenedimaleimide and dimethyl suberimidate Heimark et al. (1976) have shown

that IF3 binds in covalent crosslinked complexes with S1, S11, S12, S13, S19, and S21. Hawley et al. (1974) have used dimethyl suberimidate as a crosslinking reagent to study how IF3 promotes the disassociation of 70S ribosomes into subunits. These investigators suggest that IF3 causes a conformational change in the 70S structure to allow proper message binding. The nature of the structural change probably involves an opening at the 30S-50S interface as if the subunits were hinged together. When the ribosome is crosslinked the topography of the ribosome is fixed and IF3 cannot open the 70S structure to allow proper message binding.

C. Nucleosomes

Recent developments have suggested that the structure of chromatin is based on a linear array of subunits (Olins and Olins, 1974; Weintraub and Van Lente, 1974). The x-ray data which had previously been thought to reflect a super-coil model have now been reinterpreted in the context of this new subunit model of histone clusters as a fundamental structural feature of chromatin (Kornberg, 1974; Weintraub and Van Lente, 1974). The general picture which has emerged is that two each of histones F2a1, F2a2, F2b, and F3 associate strongly with one another forming a globular structure over or around which are arranged approximately 180 base

pairs of DNA. Preliminary studies on eukaryotic histones suggested that specific crosslinking reagents would be useful in determining the proximity of one type of histone to another.

Some of the bifunctional reagents which have been used to study the arrangement of proteins in ribosomes and in subunits of oligomeric enzymes have also been applied to isolated histones in solution and to histones in chromatin. Martinson and McCarthy (1975) studied the arrangement of histones in chromatin using the crosslinking agent tetranitromethane. The mechanism by which this reagent crosslinks proteins is not completely clear; nevertheless, the primary site of action is tyrosine. Using tetranitromethane Martinson and McCarthy found the rapid formation of a single crosslinked histone product with the molecular weight of a dimer. This crosslinked product was formed by treatment with tetranitromethane of either growing cells in culture, isolated chromatin, or reconstituted nucleohistone. On the basis of reconstitution of DNA with separated radioactive histones, the crosslinked product was identified as an F2b-F2a1 dimer. Qualitatively the results of Martinson and McCarthy (1975; 1976) supported the implications drawn by D'Anna and Isenberg (1974) from their data on histone-histone interactions in solution. They determined that the

most significant interactions were F3-F2a1, F2a1-F2b, and F2b-F2a2 and suggested that all four histones participated as an uninterrupted functional unit in chromatin. The tetranitromethane crosslinking data demonstrated very directly that, although the F3-F2a1 and F2b-F2a2 domains were apparently distinct, they were nevertheless contiguous and specifically interjoined in the chromosome (Martinson and McCarthy, 1975).

Kornberg and Thomas (1974) found pair-wise associations of the histones in solution. Two types of histone formed a tetramer, $(F2a1)_2(F3)_2$, and two other types of histone formed an oligomer of F2a2 and F2b. F1 did not crosslink. The crosslinking experiments were carried out by the method of Davies and Stark (1970) which involves (1) treating the protein with dimethyl suberimidate, a bifunctional imido-ester, and (2) determining the molecular weights of the products by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Crosslinking a mixture of the tetramer of F2a1 and F3 and oligomers of F2a2 and F2b appeared to be the sum of the individual mixtures although many bands overlapped. Evidently no additional oligomers were formed. Therefore, the evidence presented by these investigators show that the histones from chromatin of eukaryotes appear to be associated in pairs in solution, F2a1 with F3 and

F2a2 with F2b, (Kornberg, 1974).

The trend from these different types of studies indicates that, in general, all four histones are intimately associated with one another and, as a group, produce one very elementary level of chromosome packaging.

II. Macromolecular Structures of Extended Size

A. Tropomyosin

Rabbit skeletal tropomyosin, a component of the thin filaments in skeletal muscle, consists of two kinds of polypeptides, α and β . They differ slightly in amino acid composition and sequence and are separable on SDS polyacrylamide gels. The α chain, which has one cysteine residue, is present in about a 3-fold excess over the β chain which contains two cysteine residues. Lehrer (1975) has crosslinked tropomyosin by oxidation of intrinsic sulfhydryl groups to yield interpolypeptide cystine bridges. This reaction requires the close proximity of two sulfhydryl groups.

B. Plasma Membrane

Davson and Danielli proposed that membranes contain a continuous hydrocarbon phase contributed by the lipid components of the membrane. Robertson later modified and refined this hypothesis into the unit-membrane hypothesis.

Singer and Nicolson in 1972 postulated the fluid-mosaic model of membrane structure. In this model the phospholipids of membranes are arranged in a bilayer to form a fluid, liquid-crystalline matrix or core. Further, the membrane proteins are largely globular with some partially embedded in the membrane, "extrinsic" proteins; and some extending across the membrane or buried in it, "intrinsic" proteins. Although this model is not satisfactory for all membranes, it does account for many observed features and properties of biological membranes.

The arrangement of the plasma membrane proteins in the erythrocyte has been examined with a variety of probes, both chemical and enzymatic. Protein distributions in the membrane have been studied at the electron microscopic level by freeze fracture and several labeling techniques, particularly with ferritin conjugates. While the resolution of the labeling procedures is good with respect to overall cellular dimensions, the results are difficult to interpret at the level of individual peptide chains. Thus the detailed arrangement of relations between peptides in the membrane is not well understood and remains to be explored by techniques involving greater resolution (Wang and Richards, 1974). Chemical crosslinking of nearby peptides represents one approach to this problem.

Theodore Steck (1972) used a series of crosslinking reagents to covalently couple neighboring polypeptides in the isolated erythrocyte membrane. Red blood cell ghosts were incubated with various crosslinking reagents. Certain SDS polyacrylamide gel bands diminished or disappeared while new bands appeared. Steck used mild reactions since extensive crosslinking produced large aggregates which could not enter the gels. In order to terminate the crosslinking reactions, the membrane polypeptides were dissolved in SDS. Steck found no crosslinking in the presence of the detergent presumably because it rapidly abolished the native juxtaposition of the polypeptides. The entire reaction mixture could then be electrophoresed directly. Washing the membranes, which risks losing protein constituents, was not needed nor was adding inhibitors which could reverse the crosslinking reaction or alter the gel patterns. Steck found that each crosslinking reagent was consistent in its effect. He concluded that covalent crosslinking of the polypeptides offers a way of fixing interchain associations in the membrane before analysis in a dissociating detergent system. In addition, Steck pointed out that the absence of a crosslinking reaction does not rule out the association of polypeptides in an oligomer, since chemical and steric requirements must be satisfied for coupling to occur

(Wold, 1967; Davies and Stark, 1970). This was supported by the fact that no single crosslinking agent brought about all the transitions observed.

It has been well documented that red cell membranes contain two species of polypeptide chains of very high molecular weight. These two species comprise approximately one-quarter or more of the total protein and can be extracted into media of low ionic strength. The two species can be separated by SDS polyacrylamide gel electrophoresis. Their molecular weights are estimated as 225,000 and 250,000 daltons. Both species appear not to contain carbohydrate. Marchesi et al. (1969; 1970) have shown that these "spectrin" components, when the ionic strength of the solution is raised, form filamentous structures. In the absence of denaturants, the two high molecular weight chains do not separate. Hulla and Gratzer (1972) cross-linked ghosts from human red cells using the crosslinking reagent dimethyl suberimidate. They found that increasing exposure of ghosts to dimethyl suberimidate caused a progressive appearance of zones in the high molecular weight region of polyacrylamide gels with diminution in concentration of the original spectrin components. Their results further indicated that the two high molecular weight polypeptide chains of the red cell membrane were associated

with one another as subunits of an aggregate.

Glycoproteins have been shown to be vital components in determining the structure and organization of plasma membranes and there is evidence suggesting their role in cell to cell contact, adhesion, hormone interaction, and viral transformation. Three major species of glycoproteins have been found in human erythrocyte membranes. Interactions with other membrane components may be important in determining their lateral movement (Ji, 1974). Chemical crosslinking of the glycoproteins to adjacent molecules is one approach to studying their organization and interactions in the membrane. A variety of bifunctional crosslinking reagents have been tested but none of these reagents have been successful in crosslinking glycoproteins, although most of the proteins in the membrane have been crosslinked by this technique (Steck, 1972). Ji (1974) showed that although the glycoproteins cannot be crosslinked to one another, both glycoproteins and proteins can be crosslinked with dimethyl adipimidate with the formation of new glycoprotein complexes.

Frey and Shelton (1975) have examined the plasma membrane of human erythrocytes stored under blood bank conditions. These investigators utilized mild glutaraldehyde crosslinking of intact erythrocytes followed by SDS

polyacrylamide gel electrophoresis of the purified membrane proteins. The purpose of the study was to investigate alterations in erythrocyte membrane polypeptide spatial relationships that might occur in concert with gross cell shape changes. The results indicated that the major polypeptides in the plasma membrane undergo few spatial or conformational changes during storage of the blood under blood bank conditions.

In many membrane crosslinking studies the reagents used have produced stable covalently linked products. For further analysis, the crosslinked complexes have to be isolated as such and the observed molecular weight interpreted on the basis of prior knowledge of the system or with the aid of prior labeling of specific components. In general, with complex systems, the mixtures produced by partial crosslinking complicate already difficult separation problems. In an effort to overcome this analytical difficulty Wang and Richards (1974) synthesized a cleavable bifunctional reagent which contains a disulfide group. In addition, these investigators used a gel electrophoretic "diagonal mapping" technique based on the procedures used in peptide sequence analysis. The SDS gel procedure was suitable for membrane-derived polypeptides. The crosslinking reagent synthesized was dimethyl-3-3'-dithiobis-

propionimidate dihydrochloride.

Crosslinks were also effected by oxidation of the intrinsic protein sulfhydryl groups. The oxidant was (orthophenanthroline)₂Cu(II) complex. Each of these reactions results in only a very minor change in net charge in the neutral pH range for the proteins affected and both of the reagents react with a high degree of group specificity. (Wang and Richards, 1974; Lomant and Fairbanks, 1976; Kobashi and Horecker, 1967; Kobashi, 1968). The results revealed that a number of erythrocyte membrane proteins are crosslinked by simple oxidation of intrinsic sulfhydryl groups. Close proximity of two sulfhydryl groups is essential for disulfide formation to occur. The fast rate and the high yield of crosslinked products for at least some of the major polypeptides strongly suggest that adjacent sulfhydryl groups are present near the interfaces of stable, associated peptide chains. Wang and Richards found that spectrin, of all the membrane components, was the most rapidly crosslinked by both the reagents used. In addition, some of their findings supported earlier conclusions of Hulla and Gratzer (1972) and Steck (1972).

C. Nuclear Envelope

Unlike the plasma membrane, the nuclear envelope is an entirely intracellular structure. It divides the cell into

two environments; the cytoplasm and the nucleoplasm.

Evidence that the nuclear envelope is an organized structure with detectable mechanical strength come from simple experiments in micro-dissection. Callan and Tomlin (1950) demonstrated that if one attempts to puncture a cell such as an Amoeba or salivary gland cell of Drosophila, the needle meets resistance at the plasma membrane; having overcome this resistance, the needle moves relatively freely in the cell interior until it meets the nuclear membrane. Here further resistance is encountered; once the nuclear membrane has been punctured, the needle again moves relatively freely inside the nucleus.

In some nuclear types a "honeycomb layer" of proteinaceous material lies against the inner nuclear membrane over the entire nuclear surface, especially in very large nuclei. A thinner more amorphous layer occurs at the inner nuclear membrane in many other cell types. In some of these cases a fibrous lamina has been observed. These substructures seem to serve as a supportive skeleton for the nuclear envelope. Whether the chromosomes are attached to the inner membrane apposed protein layer or to the inner nuclear envelope itself remains a controversy. In 1974, Berezney and Coffey observed that isolated rat liver nuclei maintained a stable residual nuclear structure following a

wide variety of chemical treatments and extractions. These treatments removed many of the major components of the nucleus. The residual structure was disrupted however by mild digestion with proteases. This observation indicated that the nucleus contained a residual protein matrix which provided a framework structure for maintaining the shape of the nucleus. Berezney and Coffey found, after treatments to remove essentially all of the chromatin, DNA, RNA, and phospholipid, a spherical particle representing the residual nucleus composed of 98% protein. Further characterization revealed the framework structure to consist predominantly of three major acidic polypeptides of molecular weight 60-70,000 daltons as well as several minor polypeptides. Berezney and Coffey concluded that the protein matrix might play a fundamental role as the major structural component of the cell nucleus.

Aaronson and Blobel (1975) reported the isolation and partial characterization of a subfraction containing nuclear pore complexes in association with a lamina obtained from isolated rat liver nuclei. It appeared to these investigators that the pore complexes were interconnected and oriented by the lamina. They found that nuclear ghosts remained after treatment of the material with 5% Triton X-100 which they speculated to dissolve the membranes. The

Triton dissolved over 95% of the original phospholipid but dissolved only approximately 30% of the protein. The detergent-solubilized protein presumably consisted of membrane and ribosomal proteins. The Triton-insoluble residue contained three major bands of approximately 66,000, 68,000 and 69,000 daltons molecular weight. These major bands also appeared to be present in the same relative amounts as they were in the original nuclear envelope fraction while the levels of other bands initially present were reduced. Aaronson and Blobel felt that isolation of the material from a nuclear envelope fraction, as well as the asymmetric presence and common orientation of the pore complexes on the lamina, all supported the idea that the lamina derives from the nuclear periphery. Their model is consistent with the model wherein the inner and outer membrane are separated along their surface by the perinuclear space except at the nuclear pores where the two membranes show a direct continuity. They suggested that an amorphous lamina of varying dimensions depending on the cell type and possibly the metabolic state is present immediately beneath the inner nuclear membrane and that in some cell types or under certain conditions this layer may be obscured by dense heterochromatin. In these cases the lamina presence might be indicated by an apparent thickening of the inner membrane.

The nuclear pore complex is postulated to fill the nuclear pore, overlapping the margin of the pore on the outside and being continuous with the amorphous lamina on the inside. The function of the lamina would be to provide a more or less rigid skeleton, and to spatially organize the nuclear pore complexes. Aaronson and Blobel suggest that the lamina, although not always easily made visible, might be a ubiquitous component of nuclei much like the nuclear pore complexes.

Scheer et al. (1976) performed an ultramicroscopic study of the disintegration of nuclear envelopes isolated from amphibian oocytes and rat liver tissue. After treating the membranes with various detergents, they noted that residues from the detergent treatment were not released as isolated pore complex particles but that nonmembranous nuclear pore complex constituents were obtained in a form that sedimented at a low speed during centrifugation. They determined that the inefficiency in separating the individual pore complexes was due to connections between the columns of nonmembranous pore complex material that resisted the detergent treatments. The nonmembranous components of the pore complexes of nuclear envelopes were markedly resistant to membrane disintegrating forces whether mechanical or induced by the interaction of membrane components with

detergent molecules. Both kinds of forces resulted in a more or less preferential disintegration of the outer nuclear membrane. Scheer et al. postulate that such inner nuclear membrane-apposed layers act as stabilizing peripheral proteins that confer an enhanced resistance to mechanical stress and to the lipophilic action of detergents on the integral membrane proteins with which they are associated.

I have applied protein crosslinking to a study of associations among proteins in the avian erythrocyte nuclear membrane in an effort to reveal interchain associations that exist in the membrane before separation by a dissociating detergent system. The mature avian erythrocyte should be a favorable cell for the chemical crosslinking of nuclear envelope polypeptides because the cells contain predominantly plasma and nuclear envelope membranes. The nucleus appears to contain only the nuclear envelope and condensed chromatin. Thus the use of chicken erythrocyte nuclear envelope in the crosslinking studies minimizes potential membrane contaminants and simplifies interpretation of the results.

Chicken erythrocyte nuclear envelope preparations free of plasma membrane and chromatin contamination have been described by Shelton et al. (1976). With this method of

envelope isolation (Method 1 in Materials and Methods) it has been shown that much of the chicken erythrocyte nuclear envelope protein occurs in an electrophoretic band of approximately 77,000 daltons. The relationships among the polypeptides within the envelope, however, are not known. I have used a series of crosslinking reagents to covalently couple neighboring polypeptides in the isolated nuclear envelope. An alternative method (Method 2 in Materials and Methods) for isolating a similar polypeptide fraction is by a modification of a method by Dwyer and Blobel (1976).

Bifunctional small molecule reagents have been important in the study of a number of problems in protein chemistry (Dutton, Adams, and Singer, 1966). For these purposes, it is desirable that the chemical modifications produced by the bifunctional reagents cause as little alteration as possible in the conformation of the protein molecule. The exhaustive amidination of lysine epsilon-amino groups with monofunctional imidoesters preserves the positive charges near these sites and was shown to produce little or no significant changes in the conformational properties or biological activities of proteins (Hunter and Ludwig, 1962). It was therefore anticipated that bifunctional imidoesters would be highly useful crosslinking agents, in that they would not affect the charge distri-

bution of the proteins which they modified. Since then crosslinking with bifunctional reagents has been used in the investigation of macromolecular assemblies for a variety of purposes (Hajdu, Bartha, and Friedrich, 1976). The increasing popularity of this approach results from its ease and simplicity, as well as being the only chemical means to examine in detail the topography of macromolecular aggregates.

In the present study, the crosslinking results, as revealed by SDS polyacrylamide gel electrophoresis, are applied to the topography of a macromolecular structure, the avian erythrocyte nuclear envelope. It is of specific interest in this study to determine the disposition of these nuclear envelope polypeptides. A study of the proteins of the envelope provides basic information for our understanding of the molecular events which occur with this structure. Crosslinking agents which can be cleaved are also utilized. These agents allow crosslinked products to be reduced to their monomeric components. Finally, efforts are made to form disulfide crosslinks by oxidation of intrinsic sulfhydryl groups. This method, requiring adjacent sulfhydryl groups, has the potential advantage of great selectivity if such groups are appropriately located in the membrane.

MATERIALS AND METHODS

Materials

Acrylamide, Eastman Kodak Co.

Ammonium persulfate, Canal Industrial Corp.

Coomassie Brilliant Blue R, Sigma Chemical Co.

Deoxyribonuclease 1, Sigma Chemical Co.

Dimethyl adipimidate dihydrochloride, Pierce Chemical Co.

Dimethyl suberimidate dihydrochloride, Pierce Chemical Co.

Dithiobis (succinimidyl propionate), Pierce Chemical Co.

N-ethyl maleimide, Sigma Chemical Co.

N,N¹-Methylenebisacrylamide, Eastman Kodak Co.

Methyl-4-Mercaptobutyrimidate hydrochloride, Pierce Chemical
Co.

2-Mercaptoethanol, Eastman Kodak Co.

Mechlorethamine hydrochloride, Merck Sharp and Dohme

1,10-Phenanthroline, Sigma Chemical Co.

Sodium dodecyl sulfate, BDH Chemicals Ltd.

N,N,N¹,N¹-Tetramethylethylenediamine, Eastman Kodak Co.

Triethanolamine, Fisher Scientific Co.

Tris (hydroxymethyl) aminomethane, Sigma Chemical Co.

All other chemicals were reagent grade.

Collection and Washing of Cells

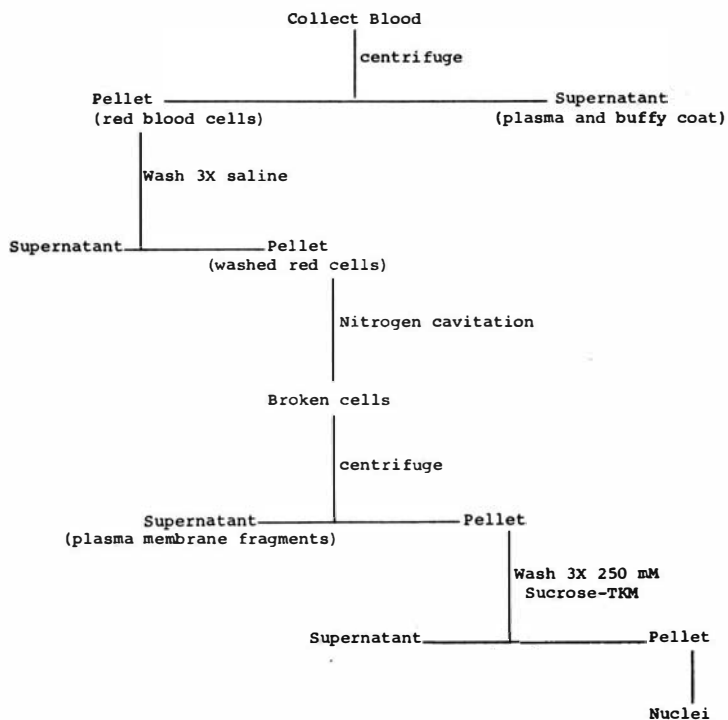
Whole blood was removed from mature chickens by heart puncture. A 3.8% solution of sodium citrate was used as an

anticoagulant. Approximately 2.0 ml of cold anticoagulant was used per 15 ml of whole blood. The blood was centrifuged in 15 ml conical centrifuge tubes for 10 min at 600 x g in an International centrifuge. Following centrifugation the buffy coat and plasma layers were removed by aspiration. The red blood cells were washed three times with 10 volumes of 146 mM NaCl. The cells were collected after each wash by centrifugation for 5 min at 270 x g using an SS34 rotor in a Dupont Sorvall RC-5 superspeed refrigerated centrifuge. The clear supernatant was removed by aspiration from the pelleted red cells. The pellet of washed red blood cells was suspended in 1-2 blood volumes of 146 mM NaCl, 1.0 mM CaCl_2 and the hematocrit was determined.

Collection and Washing of Whole Nuclei

146 mM NaCl, 1.0 mM CaCl_2 was added to give a 20-40 fold cell volume solution. The diluted cells were subjected to nitrogen cavitation in a Parr bomb for 20 min at 1000-1100 psi. Following cavitation 150-200 ml of the homogenate was underlaid with 20 ml of 20% glycerol, saline, 1.0 mM CaCl_2 . The underlaid homogenate was centrifuged for 20 min at 450 x g in an HS4 rotor. The supernatant was aspirated off and the pellets collected. The pellets were washed three times by gentle homogenization in 50 mM Tris-HCl (pH 7.5), 25 mM KCl, 5 mM MgCl_2 (TKM), 250 mM sucrose in a

Fig.1. Flow diagram for the preparation of nuclei from whole blood.



40 ml Dounce homogenizer with a loose fitting pestle and collected by centrifugation for 5 min at 480 x g. Following each centrifugation the supernatant was discarded and the pellet of nuclei collected.

Preparation of Nuclear Envelope Fraction (Method 1)

The pellet of nuclei was resuspended in TKM. A 1.0 mg/ml pancreatic DNase-TKM solution was added to the re-suspended nuclei to give a final concentration of 10 mg/ml DNase. This reaction proceeded 20 min at 4°C. Potassium citrate was added, its weight equal to 1/10 the volume of the solution. This suspension was centrifuged in a 50.2 Ti fixed angle rotor in a Beckman Model L5-50 ultracentrifuge for 30 min at 220,000 x g. The high speed pellets were resuspended with a 40 ml Dounce homogenizer with a loose fitting pestle in TKM, 250 mM sucrose. This nuclear homogenate was digested overnight at 4°C in TKM, 250 mM sucrose, 1% pancreatic DNase. The following day the digest was made 0.5 M $MgCl_2$ by the addition of an equal volume of 1.0 M $MgCl_2$. After complete mixing this solution was centrifuged 40 min at 27,000 x g. The pellet was washed in TKM, 250 mM sucrose by homogenization with a loose fitting Dounce homogenizer and centrifuged 20 min at 27,000 x g. The supernatant solution was discarded and the nuclear envelope fragments collected as pellets. The purified

envelope fraction was washed once in 50 mM triethanolamine (TEA)-HCl (pH 7.5), 25 mM KCl, 5 mM $MgCl_2$ (TEA, KM). The washed envelope fraction was collected as a pellet by a 20 min 27,000 x g spin.

Preparation of Nuclear Envelope Fraction (Method 2)

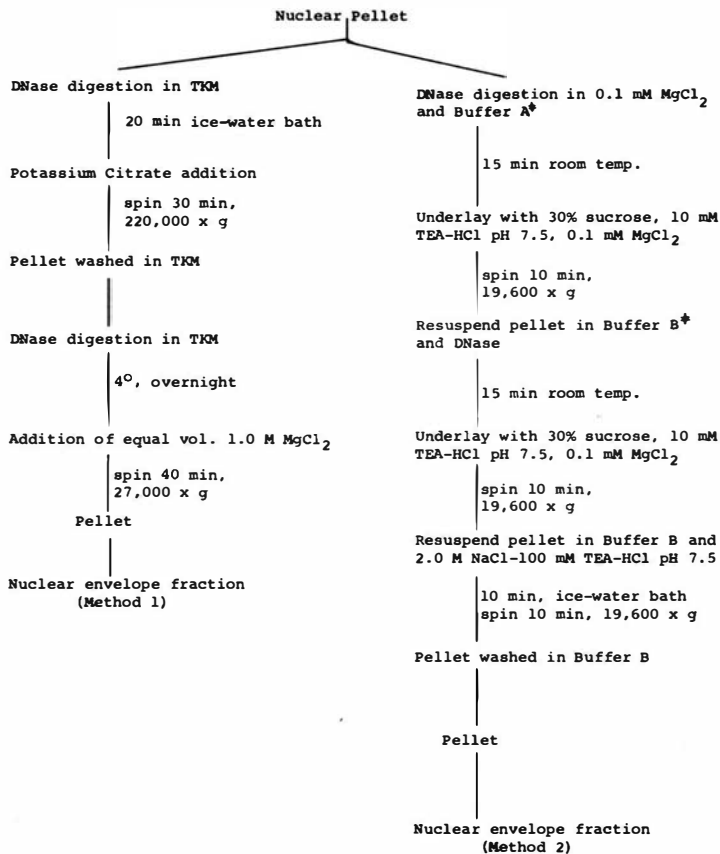
The pellet of nuclei was resuspended by homogenization in a 40 ml Dounce homogenizer with a loose fitting pestle. Five ml of 0.1 mM $MgCl_2$ was added (all volumes per ml of cell equivalent). After resuspension, 250 μ l of DNase (100 μ g/ml) was added. Finally 20 ml of 10% sucrose, 10 mM TEA-HCl (pH 8.5), 0.1 mM $MgCl_2$ was added. This mixture incubated 15 min at room temperature. After incubation, the mixture was underlaid with 5.0 ml of 30% sucrose, 10 mM TEA-HCl (pH 7.5), 0.1 mM $MgCl_2$. This solution was centrifuged at 4°C for 10 min at 19,500 x g in an HB4 - Dupont swinging bucket rotor. The pellet was resuspended in 5.0 ml of 10% sucrose, 10 mM TEA-HCl (pH 7.5), 0.1 mM $MgCl_2$. Following resuspension 250 μ l of DNase (100 μ g/ml) was added. This incubated at room temperature for 15 min. After incubation the mixture was underlaid with 30% sucrose, 10 mM TEA-HCl (pH 7.5), 0.1 mM $MgCl_2$. This solution was centrifuged at 4°C for 10 min at 19,600 x g. The resulting pellet was resuspended by homogenization in 5.0 ml of ice-cold 10% sucrose, 10 mM TEA-HCl (pH 7.5), 0.1 mM $MgCl_2$.

To this suspension was added 5.0 ml of 2.0 M NaCl and 100 mM TEA-HCl (pH 7.5). This mixture incubated 10 min in an ice-water bath and centrifuged at 4°C for 10 min at 19,600 x g. The resulting purified nuclear envelope pellet was washed once in 5.0 ml of 10% sucrose, 10 mM TEA-HCl (pH 7.5), 0.1 mM MgCl₂. This was centrifuged for 10 min at 19,600 x g. The supernatant solution was discarded and the resulting washed nuclear envelope fraction collected as a pellet.

Crosslinking Reactions With Dimethyl Adipimide Dihydrochloride and Dimethyl Superimide Dihydrochloride

The nuclear envelope fraction from 1.0 ml of packed erythrocytes was suspended in 1.0 ml of the TEA, KM buffer for each test solution. The bifunctional imide ester was prepared as a 50 mM solution in 200 mM TEA-HCl (pH 7.5). Reduced concentrations of the reagents were prepared by serial dilution in water. After 5 min at room temperature, 1.0 ml of the reagent solution was added to each 1.0 ml of envelope suspension and the reaction allowed to proceed for 20 min at room temperature. The reaction mixture was chilled in an ice-water bath and dialyzed overnight at 4°C against 100 volumes of distilled water. For electrophoretic examination, concentrated reagents were added to the dialyzed samples to constitute sample for electrophoresis.

Fig.2. Flow diagram for the preparation of the nuclear envelope fraction. Method 1 is taken from Shelton et al. (1976); Method 2 from Dwyer and Blobel (1976). Buffer A is composed of 10% sucrose, 10 mM TEA-HCl (pH 8.5), 0.1 mM MgCl_2 ; Buffer B is composed of 10% sucrose, 10 mM TEA-HCl (pH 7.5), 0.1 mM MgCl_2 .



Crosslinking Reactions With Methyl-4-Mercaptobutyrimidate

The nuclear envelope fraction from 1.0 ml of packed erythrocytes was suspended in 0.5 ml of TEA, KM to constitute each test solution. Each test solution was dialyzed 1.0 hour against 20 times the cell volume of TEA, KM, 1% 2-mercaptoethanol at 4°C. The crosslinking reagent was prepared as a 500 mM solution in 500 mM TEA-HCl (pH 7.5). To each 0.5 ml of envelope suspension 0.01 ml of reagent solution (10 mM final concentration) was added and the reaction was allowed to proceed for 20 min at 4°C. This reaction mixture was dialyzed for three hours against 100 times the cell volume of the TEA, KM buffer at 4°C. Following dialysis each reaction mixture was incubated at room temperature for 5 min after which hydrogen peroxide (40 mM final concentration) was added. After 30 min at room temperature this mixture was chilled in an ice-water bath. Concentrated reagents were added to the test solutions to constitute sample for electrophoresis.

Crosslinking Reactions With Dithiobis (succinimidyl propionate)

The nuclear envelope fraction from 0.5 ml of packed erythrocytes was suspended in 1.0 ml of 10% sucrose, 10 mM TEA-HCl (pH 7.5), 0.1 mM $MgCl_2$ to constitute each test solution. The crosslinking reagent was made as a 1.0 M

solution with dimethyl sulfoxide. From this 1.0 M solution the desired concentrations of the reagent were diluted in 1.0 ml of TEA-HCl (pH 7.5). After 5 min at room temperature, crosslinking was initiated by adding reagent solutions to nuclear envelope suspensions and the reactions allowed to proceed for 20 min at room temperature. Each reaction mixture was placed in an ice-water bath and immediately dialyzed against 100 times the cell volume of distilled water at 4°C overnight. The next day the dialyzed material was centrifuged at 4°C for 20 min at 27,000 x g. The supernatant was discarded and concentrated reagents were added to the pellets to constitute sample for electrophoresis.

Crosslinking Reactions With α -phenanthroline/CuSO₄

Nuclear envelope from 0.5 ml of packed erythrocytes was suspended in 0.9 ml of 10% sucrose, 10 mM TEA-HCl (pH 7.5), 0.1 mM MgCl₂ to constitute each test solution. The α -phenanthroline/CuSO₄ was dissolved in water to the desired concentration. Reduced concentrations of the reagent were prepared by serial dilution in water. After 5 min at room temperature, 0.1 ml of the reagent solution was added to each 0.9 ml of envelope suspension and the reaction allowed to proceed for 20 min at room temperature. Following incubation each sample was centrifuged for 20 min at 27,000 x g. The supernatant solution was discarded

and concentrated reagents were added to pellets to constitute sample for electrophoresis.

Some test solutions were treated with N-ethyl maleimide after the first washing of the whole nuclei. The pellet of nuclei was resuspended in a Dounce homogenizer in TKM, 250 mM sucrose, 10 ml per 0.5 ml of cell equivalent. N-ethyl maleimide (dissolved in ice-cold absolute ethanol) was added to give a final concentration of 1.0 mM. The mixture was incubated 5 min in an ice-water bath and was then centrifuged for 5 min at 480 x g. These nuclei were washed twice in TKM, 250 mM sucrose as described above.

Some test solutions of nuclear envelope were washed with Triton X-100. In these cases 0.5 ml of cell equivalents was suspended by homogenization in a Dounce homogenizer in 0.5 ml of 10% sucrose, 10 mM TEA-HCl (pH 7.5), 0.1 mM $MgCl_2$. To this was added 0.1 ml of 5% Triton X-100 and the mixture was extracted on ice for 20 min. The Triton-washed nuclear envelope fraction was collected by centrifugation for 20 min at 27,000 x g.

Some test solutions when not directly dissolved in sample buffer were stored under 2.0 ml of 90% ethanol overnight at $-20^{\circ}C$. The following day these samples were centrifuged for 20 min at 27,000 x g. The supernatant was placed on ice and examined for protein by the addition of

3.0 ml of ether. Concentrated reagents were added to the pellets to constitute sample for electrophoresis.

Polyacrylamide Gel Electrophoresis

For electrophoretic examination, concentrated reagents were added to the dialyzed samples to constitute sample for electrophoresis consisting of 10 mM TEA-HCl (pH 7.5), 1.0 mM EDTA, 3% 2-mercaptoethanol, 1% SDS, 10% glycerol. The protein was dissolved by boiling for 2-3 min. Electrophoresis and staining were performed by the method of Fairbanks et al. (1971) as previously modified (Cobbs and Shelton, 1975). The Coomassie Brilliant Blue stained gels were scanned with a Gilford spectrophotometer. Some control and crosslinked nuclear envelope fragments were dissolved in sample buffer without 2-mercaptoethanol depending on crosslinking reagent and test solution.

Protein Assay

All protein was determined by a modification (Bensadoun and Weinstein, 1976) of the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Phase Microscopy

Cells and fractions were examined by phase and interference contrast microscopy.

RESULTS

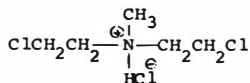
Mustard nitrogen, mechlorethamine hydrochloride (Table 1), was used in our first attempts to reveal nuclear envelope polypeptide interactions by crosslinking reactions. No polypeptides demonstrated selective reactivity although virtually all could be crosslinked by this reagent (results not shown). Because these results might reflect the variety of chemical groups reacting with the nitrogen mustard (Wheeler, 1973) rather than a lack of order among the polypeptides, more selective agents were tried.

Treatment of Nuclear Envelope With Bifunctional Imidoesters

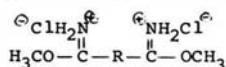
Isolated nuclear envelope (Fig.2, method 1) was exposed to varying concentrations of the bifunctional crosslinking agent dimethyl suberimide (Table 1) which reacts selectively with amino groups (Dutton et al., 1966). Reactivity of polypeptides in the nuclear envelope was assessed by dissolving the reacted envelope fraction with SDS and examining the dissolved polypeptides by polyacrylamide gel electrophoresis. Crosslinking resulted in an altered gel electrophoretic pattern. Results obtained by treating envelope with dimethyl suberimide are presented in Fig.3. As can be seen in Fig.4a, an increase in dimethyl suberimide concentration from 0 - 16.7 mM caused a selective

TABLE 1

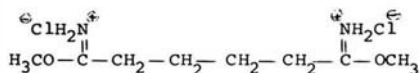
Mechlorethamine hydrochloride



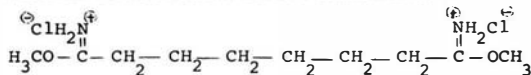
General formula for water soluble imidoesters



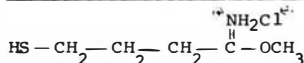
Dimethyl adipimidate dihydrochloride



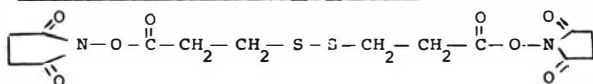
Dimethyl suberimidate dihydrochloride



Methyl 4-mercaptobutyrimidate hydrochloride



Dithiobis (succinimidyl propionate)



Dimethyl-3,3-dithiobispropionimidate dihydrochloride

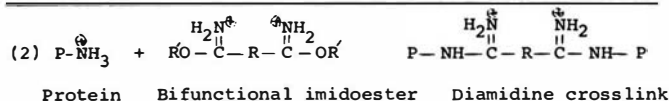
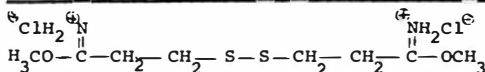
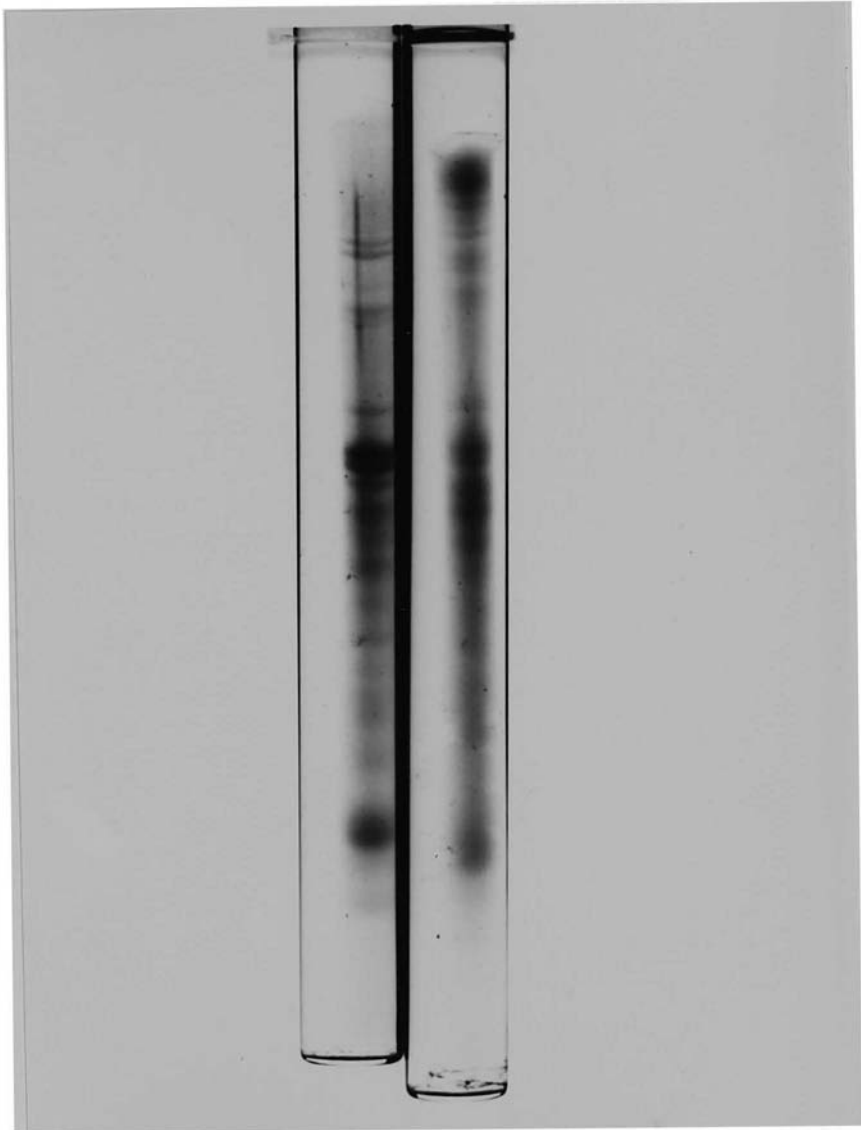


Fig.3. Electropherograms of (1) control chicken erythrocyte nuclear envelope polypeptides and (2) chicken erythrocyte nuclear envelope polypeptides crosslinked with dimethyl suberimidate dihydrochloride. Each fraction was prepared as described in the Methods section with the exception that for control polypeptides 1.0 ml water was added to the envelope suspension rather than 1.0 ml of the reagent (final concentration 8.3 mM). Envelope polypeptides from 40 μ l of packed erythrocytes were applied to each gel. The gels were 5.6% acrylamide. The arrows denote the major bands of interest: (A) major doublet characteristic of fraction (B) major crosslinked band.

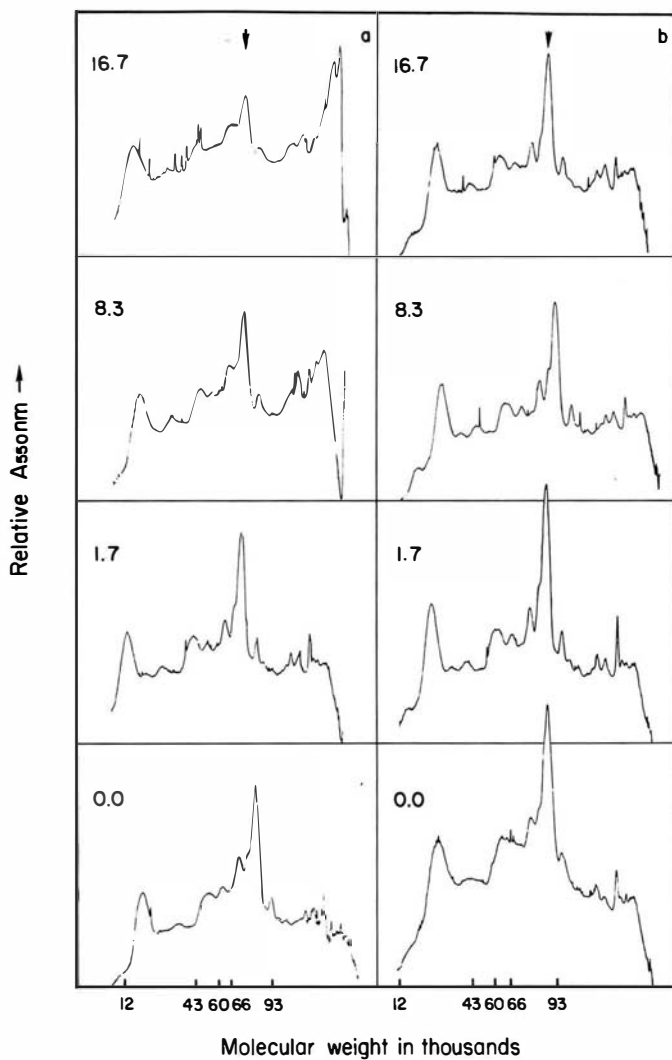
1 2



B

A

Fig.4. Electropherograms of chicken erythrocyte nuclear envelope polypeptides following exposure of the envelope fraction to various concentrations of crosslinking reagents. The two reagents used were (a) dimethyl suberimidate and (b) dimethyl adipimidate. Concentration of reagent (mM) in each test is shown in the panel with the electropherogram of the resultant polypeptides. The position of the 77,000 dalton band is indicated by an arrow. Envelope polypeptides from 40 μ l of packed erythrocytes were applied to each gel. The gels were 5.6% acrylamide. Tops of the gels correspond to the right side of each panel.

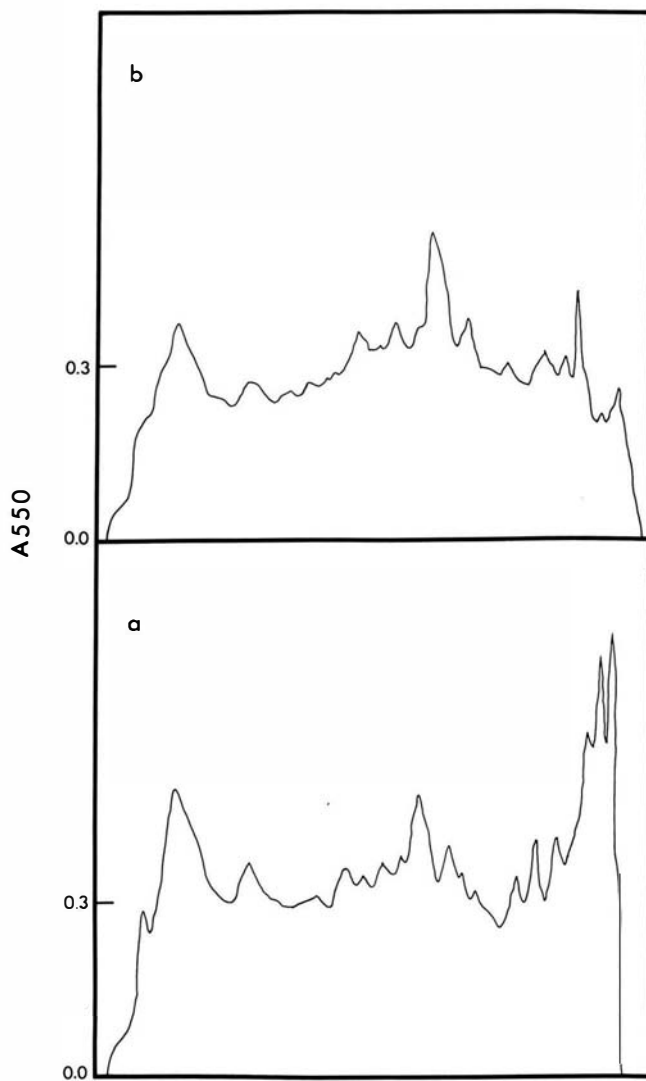


decrease in polypeptides of approximately 77,000 daltons with a concomitant increase in material greater than 200,000 daltons. At least two polypeptides can be detected in the 77,000 dalton band by electrophoresis on 4-30% gradient polyacrylamide gels (Cobbs and Shelton, 1975; Shelton et al., 1976) and both appear to be crosslinked (see Fig.3, arrow A). As can be seen in Fig.4b, dimethyl adipimidate at the same concentration as dimethyl suberimidate did not specifically diminish the 77,000 dalton band or give rise to the high molecular weight material. It should be noted that in dimethyl adipimidate there are two fewer methylene groups and therefore a different distance between the reactive sites (see Table 1).

Treatment of Nuclear Envelope With Cleavable Crosslinking Agents

Methyl 4-mercaptobutyrimidate (Table 1) formed amidine linkages between lysine ϵ -amino groups in polypeptides in the nuclear envelope and the imidate group of a bi-functional agent. Reaction with intrinsic sulfhydryl groups sufficiently close to form intermolecular disulfide bonds was effected by mild oxidation of the modified polypeptides. This reaction resulted in a selective decrease in polypeptides of approximately 77,000 daltons with an increase in higher molecular weight material (Fig.5a).

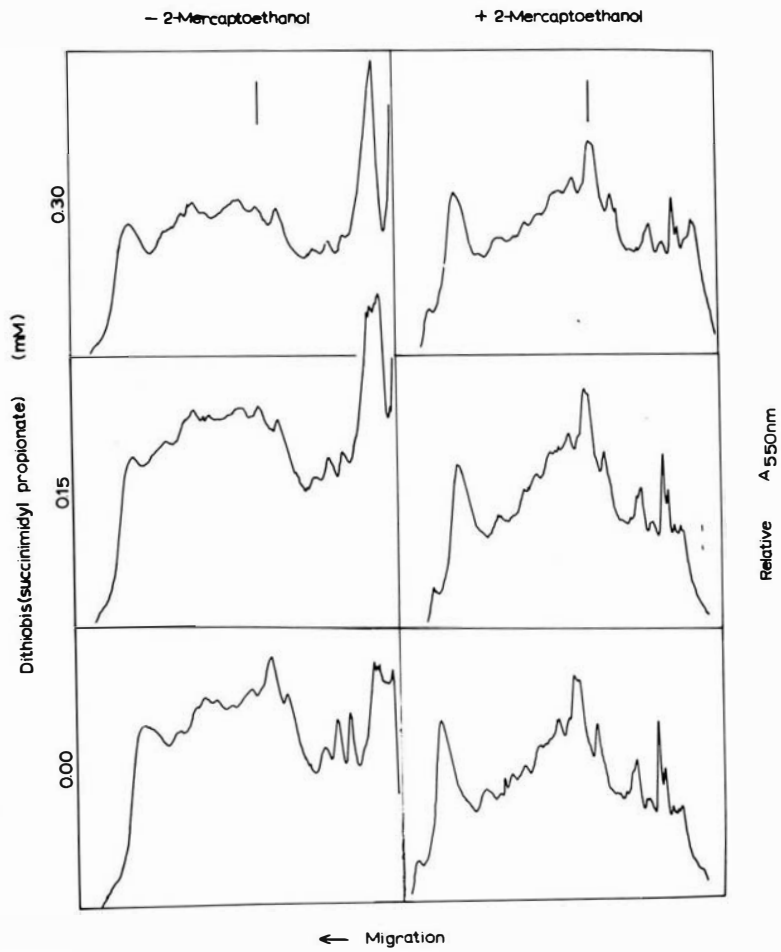
Fig.5. Electropherograms of chicken erythrocyte nuclear envelope polypeptides following exposure of the envelope to (a) methyl 4-mercaptopbutyrimidate (final concentration 10 mM) and (b) the resulting pattern obtained when the polypeptides are reduced with 3% 2-mercaptoethanol. Envelope polypeptides from 40 μ l of packed erythrocytes were applied to each gel. The gels were 5.6% acrylamide. Tops of the gels correspond to the right side of each panel.



Reduction with 2-mercaptoethanol regenerated the monomeric components of the high molecular weight crosslinked species (Fig.5b). Although this agent appeared to selectively crosslink the major nuclear envelope polypeptide species, consistent results were impossible to obtain perhaps because of reagent instability. Therefore this approach was not pursued in depth.

In searching for reproducible results with a cleavable crosslinking agent, isolated nuclear envelope (Fig.2, method 2) was exposed to varying concentrations of the cleavable bifunctional active ester dithiobis (succinimidyl propionate). This agent reacts with free primary and secondary aliphatic amino groups (Lomant and Fairbanks, 1976). Reactivity of polypeptides in the nuclear envelope was assessed by dissolving the reacted envelope fraction with SDS and examining the polypeptides by polyacrylamide gel electrophoresis. Crosslinking again resulted in an altered gel electrophoretic pattern. Preliminary results are shown in Fig.6. An increase in dithiobis (succinimidyl propionate) from 0 - 0.30 mM caused a selective decrease in the approximately 77,000 dalton polypeptides with a reciprocal increase in material greater than 200,000 daltons. Subsequent reductive cleavage of the crosslinked products by 2-mercaptoethanol in each case resulted in normal gel

Fig.6. Electropherograms of chicken erythrocyte nuclear envelope polypeptides following exposure of the envelope to various concentrations of the crosslinking reagent dithiobis (succinimidyl propionate). Concentrations of reagent (mM) are shown to the left of each set of panels of the resultant polypeptides obtained with and without 2-mercaptoethanol. The position of the 77,000 dalton band is indicated by a line. Envelope polypeptides from 65 μ l of packed erythrocytes were applied to each gel. The gels were 5.6% acrylamide. Tops of the gels correspond to the right side of each panel.

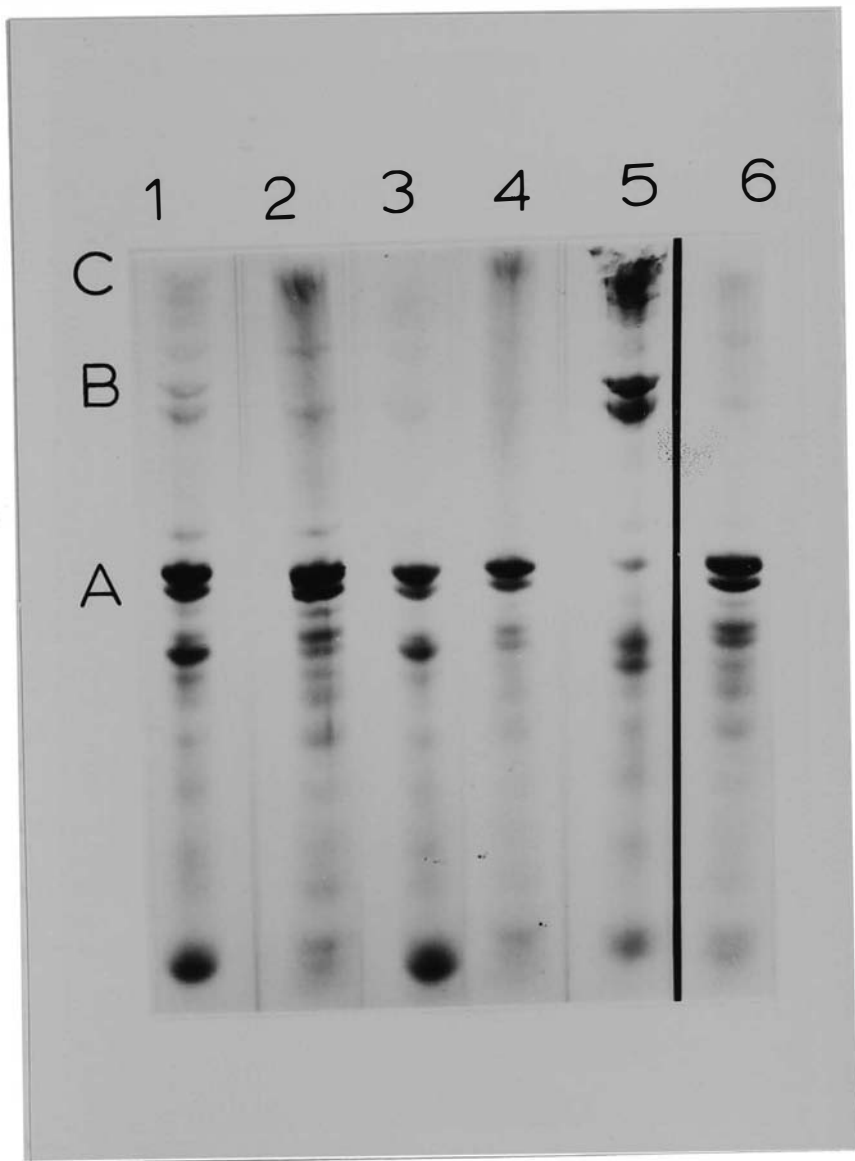


electrophoretic patterns. Identification of specific protein components in the crosslinked products by a two-dimensional gel electrophoresis system appeared to be feasible. From these preliminary studies however some interesting behavior was detected in certain polypeptide species which were not treated with any crosslinking agent.

Treatment of Nuclear Envelope With an Oxidizing Agent

Certain envelope polypeptide species (Fig.2, method 2) were present in the normal gel electrophoretic pattern in the absence of the reducing agent, 2-mercaptoethanol. This observation suggested that some crosslinks formed by sulfhydryl groups occurred in the isolated envelope. The polymers appeared to be a pair of dimers derived from the approximately 77,000 daltons pair of monomers. As shown in Fig. 7, after mercaptoethanol reduction of the material in gel 1, the larger of the pair of bands at B disappears and the smaller one is greatly reduced. Treatment of the isolated nuclei with the sulfhydryl blocking agent N-ethyl maleimide prior to envelope isolation prevented polymerization (Fig.7, gel 3 (no mercaptoethanol) and gel 4 (with mercaptoethanol)). This result confirmed the nature of the bond involved and demonstrated that in the native state the erythrocyte polypeptides were not disulfide linked. Polymerization appeared to occur during the course of envelope

Fig.7. Electropherograms of reduced, partially oxidized and oxidized chicken erythrocyte nuclear envelope polypeptides. Gel 1 reveals polypeptide crosslinking found following a standard envelope preparation. In gel 3 free sulfhydryl groups were blocked with N-ethyl maleimide after nuclei were released from the cells. In gel 5 cystine formation was promoted by exposure of isolated nuclear envelope to a solution containing o-phenanthroline/cupric sulfate (500/100 μ M). Identically treated samples in each case were reduced overnight with 3% 2-mercaptoethanol before electrophoresis (gels 2,4, and 6 respectively). Protein applied to each gel was 1 and 2: 17.4 μ g; 3 and 4: 16.2 μ g; and 5 and 6: 24.2 μ g. A indicates the position of the 77,000 daltons pair of monomers, B the position of an approximately 160,000 daltons pair of bands, and C indicates high molecular weight polymers. The gels were 5.6% acrylamide.

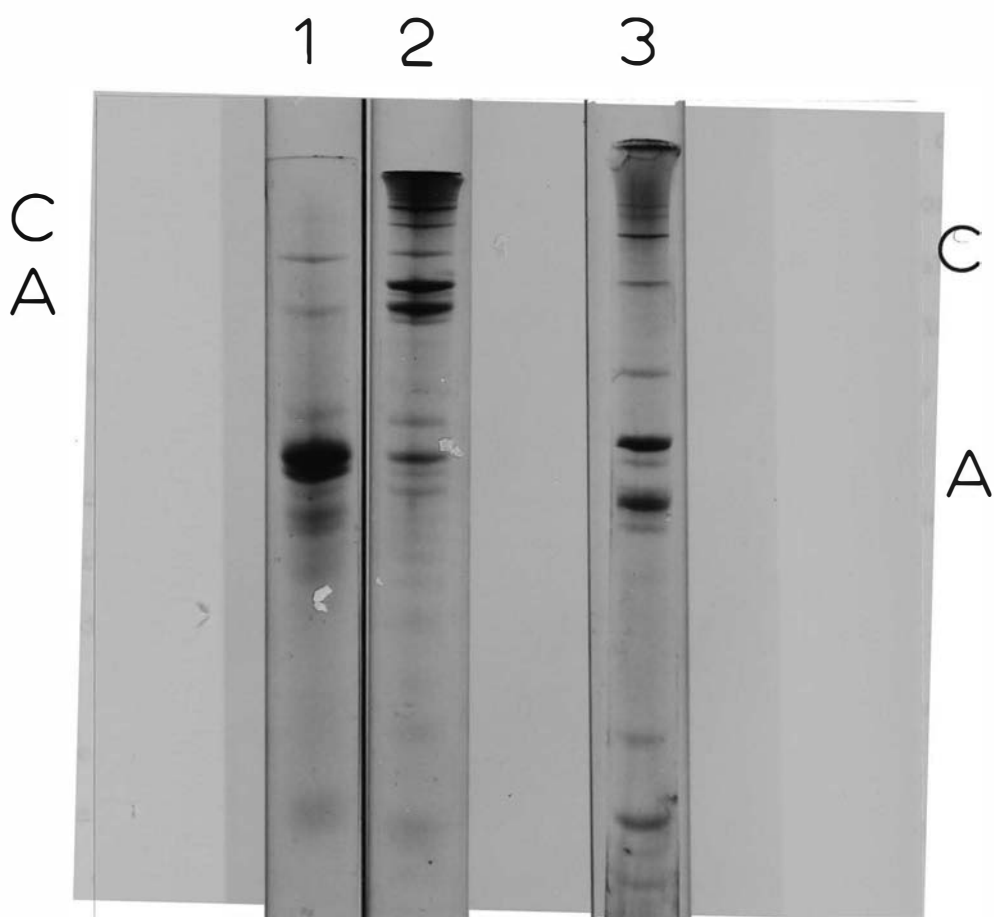


isolation.

The extent of the oxidation reaction occurring during isolation and the specificity for polypeptides were noteworthy. Phenanthroline cupric complex, a sulfhydryl group oxidant (Kobashi and Horecker, 1967; Kobashi, 1968) was tested to determine if the polymerization could be enhanced. Addition of the oxidant to a preparation of nuclear envelope resulted in a major depletion of the bands at A and an enhancement of the pair of bands at B as well as the appearance of higher polymers at C (Fig.7, gels 5 and 6).

Insolubility in solutions of nonionic detergent at low ionic strength has been a characteristic of proteins which appear to be involved in nuclear skeletons (Berezney and Coffey, 1974; Aaronson and Blobel, 1975; Scheer et al., 1976; Comings and Okada, 1976). It has also been demonstrated that the major erythrocyte nuclear polypeptides are insoluble (Shelton et al., 1975; Shelton, 1976). We therefore sought to confirm that the disulfide-crosslinked and the Triton X-100 insoluble polypeptides were identical. Two test solutions of nuclear envelope fractions treated with a mixture of o-phenanthroline and CuSO_4 and then Triton-washed are shown in Fig. 8. Bands in gel 2, area A, were selectively diminished while new bands appeared in both areas B and C. Mercaptoethanol (gel 1) restored the

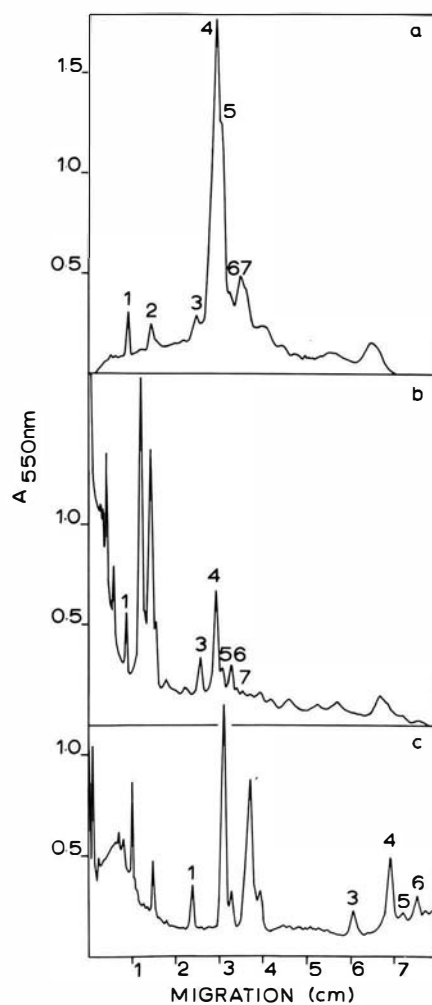
Fig.8. Electropherograms of chicken erythrocyte nuclear envelope polypeptides treated with o-phenanthroline/ CuSO_4 (500/100 μM). The polypeptides in gel 1 were reduced with 3% 2-mercaptoethanol and those in gels 2 and 3 were not. Gel 3 resulted from a doubled electrophoresis time. A indicates the position of an approximately 160,000 daltons pair of bands, and C indicates high molecular weight polymers. 14.5 μg protein was applied to each gel. The gels were 5.6% acrylamide.



normal gel electrophoretic pattern from the crosslinked species. One test solution of o-phenanthroline/CuSO₄ treated, Triton-washed nuclear envelope was dissolved in sample buffer without mercaptoethanol and electrophoresed twice as long as usual. This resulted in a clear separation of the crosslinked bands (gel 3, positions B and C).

Densitometry tracings reveal polypeptide bands more clearly by allowing comparisons to be made between peak heights and widths. Tracings of the SDS polyacrylamide gels in Fig. 8 are shown in Fig. 9. Bands 4 and 5 are the two major polypeptide species in the nuclear envelope fraction (panel a). Bands 3 and 6 are shoulders on the approximately 77,000 dalton molecular weight species. Band 7 is composed of two polypeptide species. Bands 1 and 2 are the only high molecular weight species in the envelope fraction after reduction. Bands 4 and 5 are greatly diminished when crosslinks have been produced by the catalyzed oxidation of sulfhydryl groups with the o-phenanthroline-cupric ion complex (panel b). Bands 3 and 6 do not appear to be affected. The dimer of band 7 is involved in a crosslinking reaction. The oxidation-dependent high molecular weight pair between bands 1 and 3 obscure band 2. There is also a concomitant increase in higher molecular weight material larger than band 1. When the

Fig.9. Electropherograms of o-phenanthroline/CuSO₄ (500/100 μ M), Triton X-100 insoluble nuclear envelope polypeptides after (a) mercaptoethanol reduction, (b) no reduction, and (c) extended electrophoresis of the sample in (b). The numbers refer to material which appears as distinct bands in the reduced sample. 14.5 μ g protein was applied to each gel. The gels were 5.6% acrylamide. Tops of the gels correspond to the left side of each panel.

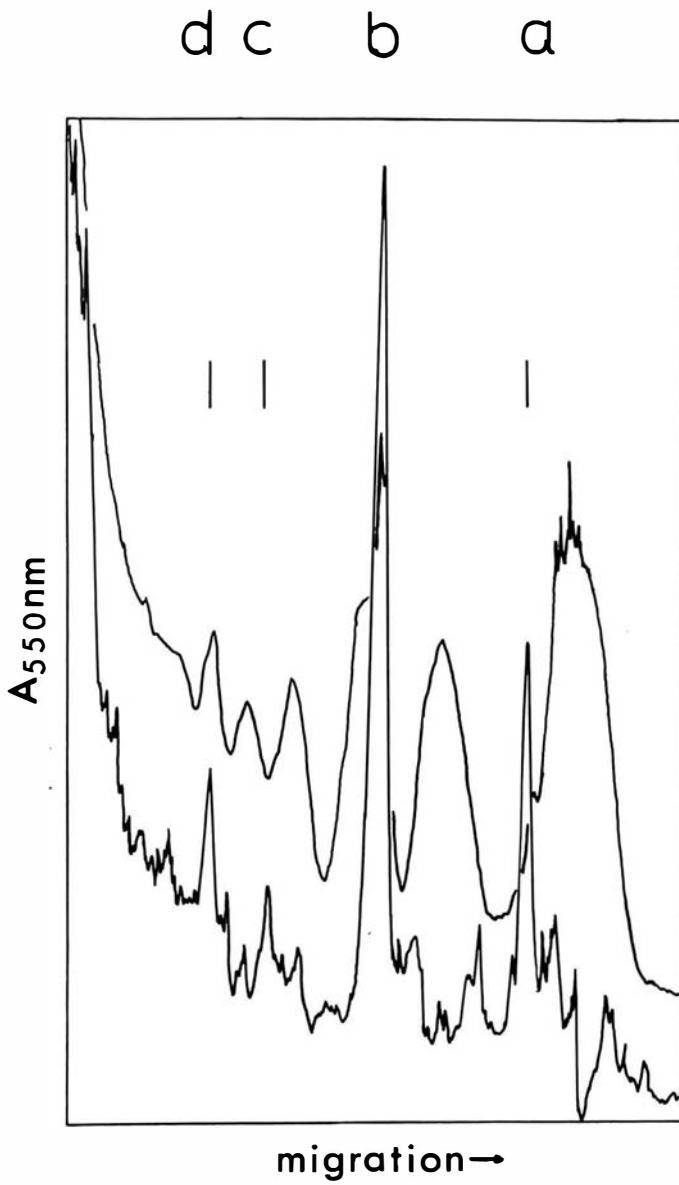


crosslinked material is run four hours on polyacrylamide gels, the crosslinked species can better be examined (panel c). The crosslinked material between bands 1 and 3 are clearly seen as two distinct species. The higher molecular weight material is resolved into 4 distinct species. Band 1 appears to be the only very high molecular weight polypeptide species that is not affected by crosslinking or reduction. It should be noted that distinct species (seen as sharp bands) predominate in the gel regions distal to 1 cm.

In Fig. 10 oxidized polypeptides are compared with standard molecular weight markers after electrophoresis on 4% polyacrylamide gels. The two dimeric species arising from oxidation do not separate on 4% polyacrylamide gels. The monomeric species appear to be approximately 75-77,000 daltons. The crosslinked dimeric species (a single band on this gel) is approximately 150-160,000 daltons. The next principal species is approximately 298-300,000 daltons. The minor species at approximately 225,000 daltons appears to be band 1 which is not involved in crosslinking. Higher molecular weight species can be detected but their molecular weights cannot be accurately determined from this experiment.

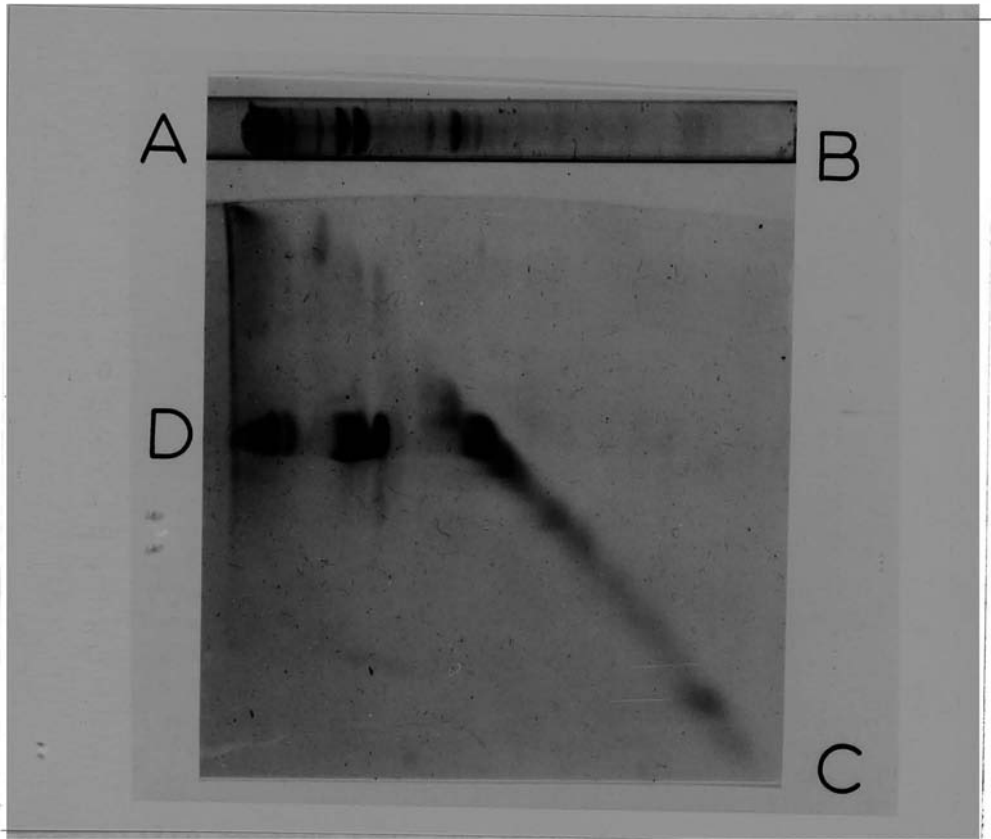
Separation and identification of crosslinked species can be obtained by a two-dimensional SDS polyacrylamide gel

Fig.10. Electropherogram of chicken erythrocyte nuclear envelope polypeptides (lower tracing) superimposed on electropherogram of molecular weight standards (upper tracing). Both gels were 4% acrylamide and 2.5% of the monomer was N,N¹-methylenebisacrylamide. Each standard molecular weight peak represents consecutive increments of 53,000 daltons. The major polypeptide species of interest are indicated by lines or a major peak at (a) 75-77,000 daltons; (b) 150-160,000 daltons; (c) 225,000 daltons; (d) 298-300,000 daltons. The nuclear envelope polypeptides represent 14.5 μ g protein. Tops of the gels correspond to the left side of each panel.



electrophoresis technique. The o-phenanthroline/ CuSO_4 treated, Triton-washed nuclear envelope polypeptides containing species crosslinked by intermolecular disulfide bonds are separated in the oxidized form by electrophoresis in SDS gels. The protein in the resulting gel is reduced and electrophoresed in an SDS-polyacrylamide slab gel. The polypeptides which have not undergone crosslinking fall on a diagonal line. Only polypeptides previously engaged in protein-protein disulfide bonds occur below the diagonal. The reduction produces monomeric envelope polypeptides which migrate more rapidly than the crosslinked complexes. In Fig.11 the crosslinked, Triton-washed polypeptides are separated in the first dimension by electrophoresis from A to B in an SDS-polyacrylamide cylindrical gel. Following incubation in a 5% 2-mercaptoethanol solution, the first dimension gel was placed on top of an identical SDS-polyacrylamide slab gel and electrophoresed from B to C. The polypeptides lying on the diagonal A to C were not involved in crosslinking. The polypeptides involved in crosslinking fell on a horizontal line passing from D to the diagonal line. The results indicate that the crosslinked material formed by the o-phenanthroline/ CuSO_4 oxidation was derived from one area on the gel corresponding to the approximately 75-77,000 dalton molecular weight polypeptide species.

Fig.11. Two-dimensional electropherogram of oxidized (1st dimension) and reduced (2nd dimension) Triton X-100 insoluble nuclear envelope polypeptides. Sample (116 μ g) was prepared and separated electrophoretically in the first dimension as described in Fig.9. A photograph of a control gel containing 14.5 μ g of protein is shown between A and B. To effect reduction the gel was immersed in 100 ml of electrophoresis buffer containing 5% 2-mercaptoethanol for 45 min. The gel, containing reduced polypeptides, was then placed at the top of a 0.35 cm x 13 cm x 10.5 cm vertical slab gel of the same composition and electrophoresed for 3 hours. A to C indicates the diagonal where polypeptides which contained no interpolypeptide cystine bonds are expected. A horizontal line passing through D and intersecting the A to C diagonal indicates the locus of most polypeptides which were crosslinked.



DISCUSSION

The major polypeptides of the chicken erythrocyte nuclear envelope have been dissociated in SDS and resolved by polyacrylamide gel electrophoresis (Shelton et al., 1976). Their interactions within the envelope were not revealed by that study. It was not known if the polypeptides were free and independent of one another, whether they interacted randomly, or were associated in specific patterns. The data reported herein suggest that some of the major components interact preferentially with identical or closely related polypeptides, while others show no such tendency.

Exposure of isolated nuclear envelope from chicken erythrocytes to dimethyl suberimidate resulted in a selective decrease in the predominant polypeptide species with a concomitant increase in higher molecular weight material. Interpolypeptide crosslinks formed by this agent reflect spatial association of the involved polypeptides. The possibility existed that the polypeptide associations indicated by these results arose randomly during the envelope isolation procedure. If so, one would expect a range of reactive site distances and therefore little or no specificity with respect to similar crosslinking reagents which varied only in chain length. The chain lengths of

the imidoesters vary with the number of methylene groups, therefore they provide an opportunity for assessing the distance between reactive sites (Dutton et al., 1966; Davies and Stark, 1970). Dimethyl adipimidate, with two less methylene groups between the bifunctional imidoester ends, when used at the same concentrations as dimethyl suberimidate did not specifically diminish the predominant polypeptide species or give rise to the high molecular weight material. This observation indicated that the predominant polypeptide species occur in a specific oligomeric arrangement in the isolated nuclear envelope. Oligomeric polypeptide associations may occur in other membranes. Hulla and Gratzer (1972) have shown that the two spectrin components in the human erythrocyte plasma membrane can be crosslinked, by dimethyl suberimidate, into dimers and at least four more higher oligomeric species. These investigators suggest that the two high molecular weight polypeptide chains are associated with one another, as subunits of an aggregate. Theodore Steck (1972) in his crosslinking studies on the erythrocyte plasma membrane noted a number of homopolymers and suggested that oligomeric proteins may be a general feature of membrane architecture. The imidoester crosslinking results reported here provide independent evidence for specific oligomeric nuclear envelope polypeptide associations.

The imidoester results were confirmed and extended by oxidation of intrinsic sulfhydryl groups. Exposure of the isolated nuclear envelope fraction to oxidizing conditions revealed that the same major polypeptides can undergo highly selective crosslinking via disulfide bond formation. The disulfide bonds are not present in the native nucleus because treatment with a sulfhydryl blocking agent before isolation of the envelope caused the disappearance of the high molecular weight material. Kobashi and Horecker (1967) originally showed that the o-phenanthroline/cupric-ion complex greatly stimulated the air oxidation of sulfhydryl groups to disulfides. The two-dimensional electrophoresis indicated that oxidation by this agent involved only the two predominant polypeptide species. The extensive crosslinking of the major polypeptide species by simple oxidation of intrinsic sulfhydryl groups can be interpreted in terms of the adipimidate and suberimidate results. These results indicated that the major polypeptide species in the nuclear envelope occurred in a specific oligomeric arrangement. Disulfide bond formation by the o-phenanthroline/cupric-ion complex requires close proximity of two sulfhydryl groups. In the oxidation of the major polypeptide species, the high yields of crosslinked products strongly suggest that adjacent sulfhydryl groups are present near the interfaces of

stable, associated peptide chains (predicted by the adipimide and suberimide results). Although these groups must be close enough to form a disulfide bridge, they must occur in areas where local movement can occur to permit reactions with N-ethyl maleimide. In vivo, this network of adjacent sulfhydryl groups must have been kept reduced since if oxidation occurred, more crosslinked products would be observed in the standard nuclear envelope SDS gel electrophoretic patterns. Thus some structural or functional role for these free sulfhydryl groups is suggested. In vitro oxidizable sulfur atoms have been observed in the fibrous protein tropomyosin where oxidation has been shown to yield α and $\alpha\beta$ dimers (Lehrer, 1975). The erythrocyte plasma membrane protein spectrin can also be covalently polymerized by cystine bond formation. In the case of spectrin, the 225,000 and 250,000 dalton polypeptides appear to form a series of polymers (Steck, 1972; Wang and Richards, 1974). Spectrin is similar to the predominant nuclear envelope polypeptide species in some solubility properties (Shelton, 1976) and a skeletal function has been suggested for it (Steck and Yu, 1973; Yu et al., 1973).

Several factors indicate that these approximately 77,000 dalton molecular weight species, which are preferentially and selectively crosslinked, may play a structural

role in the nuclear envelope. First, these species are the predominant polypeptides in the nuclear envelope fraction as prepared by two different methods. Secondly, these polypeptides are arranged as an oligomer as evidenced by these crosslinking studies. Finally, these major polypeptides are especially resistant to various washing protocols including Triton X-100 treatment (Shelton, 1976). A resistance to washing protocols has been considered evidence for proteinaceous structures. Intracellular proteinaceous structures do occur and might provide some type of skeletal support in erythrocyte plasma membranes (Yu et al., 1973) and in nuclei (Berezney and Coffey, 1974; Aaronson and Blobel, 1975).

The data presented provide new insight into specific polypeptide associations in membranes and a basis for further studies of specific details of nuclear protein architecture. The imidoester crosslinking results reported provide independent evidence for specific nuclear envelope polypeptide associations in the presence of the lipid matrix. The crosslinking via oxidation of intrinsic sulfhydryl groups support and extend the imidoester results. Adjacent sulfhydryl groups are present near the interfaces of stable associated peptide chains and exist in the membrane, at least in the case of the mature erythrocyte nucleus, as free sulfhydryl groups. In addition, the ease with which

the polymers are cleaved to the original monomers and the high yield of discrete polymeric bands, including both dimers and higher polymers, make this crosslinking agent especially useful. These oxidation results should provide a basis for future experiments on the molecular architecture of the nucleus and better our understanding of the molecular events which occur in this macromolecular structure.

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